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Species-specific Agonist/Antagonist Activities of Human Interleukin-4 Variants Suggest Distinct Ligand Binding Properties of Human and Murine Common Receptor γ Chain*

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Dominikus Bönsch, Winfried Kammer, Antje Lischke, and Karlheinz Friedrich‡

From the Theodor-Boveri-Institut für Biowissenschaften (Biozentrum), Universität Würzburg, Physiologische Chemie II, Am Hubland, D-97074 Würzburg, Federal Republic of Germany

Interleukin-4 (IL-4) is a pleiotropic cytokine eliciting various responses in target cells upon binding to its receptor. Activation of the IL-4 receptor probably involves interaction of the ligand with both the IL-4 receptor α subunit (IL-4Rα) and the common γ chain (cγ). Although human and murine IL-4 receptor α chains are specific for IL-4 from the same species, murine cγ can form a signal-competent complex with human IL-4Rα (hIL-4Rα) and human IL-4 (hIL-4). We have generated a hIL-4 responsive murine myeloid cell line (FDC-4G) expressing a chimera comprising the extracellular domain of human IL-4Rα and the intracellular domain of human granulocyte colony-stimulating factor receptor (hG-CSFR). This hybrid receptor was shown to form a complex with hIL-4 and the murine cγ-chain. Biological activities of human IL-4 variants on murine FDC-4G cells and on the human erythroleukemic cell line TF-1 displayed a strikingly different pattern. Single amino acid replacements at two different positions in the C-terminal helix of hIL-4, the region of the previously defined "signaling site," lead to an inverse agonist/antagonist behavior of the resulting cytokines in the two cellular systems. From these findings we conclude that upon formation of the activated IL-4 receptor complex murine and human cγ interact with hIL-4 in a geometrically different fashion.

Interleukin-4 exerts its activity on target cells by interaction with at least two membrane-bound receptor chains, *i.e.* the IL-4Rα[1] subunit (formerly termed IL-4R) (1), and the common receptor γ chain (cγ) (2, 3), both of which are members of the hematopoietin receptor superfamily (4). The induction of productive IL-4 receptor complex formation by binding of the ligand is not yet understood; however, mutational analysis of hIL-4 indicated that two distinct structural determinants of the cytokine are important for this process (5, 6). Amino acids located within helixes A and C of the hIL-4 molecule have been shown to be essential for the interaction with hIL-4Rα, whereas three positions in the C-terminal helix have proven crucial for signaling. Replacement of residues Arg^{121}, Tyr^{124}, or Ser^{125} yielded high affinity partial agonists or antagonists of hIL-4 in cellular assays employing human B or T cells (6), most probably by interfering with a productive interaction of hIL-4 and cγ. hIL-4 does not detectably bind to murine IL-4Rα (1, 7). Several groups have shown, however, that human IL-4Rα can confer hIL-4 responsiveness to murine lymphoid cells when expressed by gene transfer, thus indicating that murine cγ is able to form a signaling competent receptor complex with hIL-4 and hIL-4Rα (1, 8–11).

In order to investigate the interactions between hIL-4 and the two hIL-4 receptor components, we have established a hIL-4-responsive murine myeloid precursor cell line. A chimera of the extracellular domain of hIL-4Rα fused to the cytoplasmic portion of hG-CSFR was expressed in factor-dependent FDC-P1 cells and shown to become associated with murine cγ upon binding of hIL-4. Subsequently the formation of productive receptor complexes resulting in cell proliferation was studied using mutant variants of hIL-4. Comparison of the results with those obtained in a parallel set of experiments employing the hIL-4-responsive human cell line TF-1 revealed a different activity pattern of hIL-4 variants. These findings are discussed with respect to the putative interaction of hIL-4 with murine and human cγ.

MATERIALS AND METHODS

RNA Isolation, cDNA Synthesis, and Molecular Cloning—Total RNA was isolated from 1 g of fresh human placenta (obtained from Dr. J. Martius, Department of Gynecology, University of Würzburg) according to the guanidinium thiocyanate-phenol-chloroform extraction method (12), and used as a template for cDNA synthesis which was performed using Superscript Reverse Transcriptase (Life Technologies, Inc.) following the manufacturer's instructions. The cDNA clone providing the extracellular domain of hIL-4Rα has been described previously (5). Oligonucleotide syntheses, polymerase chain reactions, and other enzymatic manipulations of DNA were done following standard procedures (13). For transfection experiments SV40-based expression vector pKCR (14) and neomycin resistance plasmid pSV2neo (15) were employed. Other reagents and enzymes were from Boehringer (Mannheim, Germany), Fermentas (Vilnius, Lithuania), Amersham-Buchler (Braunschweig, Germany), and Merck (Darmstadt, Germany).

Cell Culture, Transfections, and Flow Cytometry—Both the murine myeloid precursor cell line FDC-P1 (16) and the human erythroleukemic cell line TF-1 (17) were described previously. Cells were routinely grown in DMEM, 8% FCS (FDC-P1) or RPMI 1640, 8% FCS (TF-1). Media were supplemented with 5% culture supernatant of mIL-3 producing X63Ag8–653 BPV mIL-3 cells (18) (FDC-P1) or recombinant GM-CSF basically produced as described for hIL-4 (19) at a final concentration of 5 nM (TF-1). Transfection of FDC-P1 cells was performed by electroporation using a Easyject® electroporator (Eurogentec). 3.5 ×

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‡ To whom correspondence should be addressed. Tel.: 49-931-8884124; Fax: 49-931-8884113.

[1] The abbreviations used are: IL-4, interleukin-4; IL-4Rα, the IL-4 receptor α subunit; hIL-4, human interleukin-4; hIL-4R, human interleukin-4 receptor; mIL-4, murine interleukin-4; mIL-3, murine interleukin-3; hIL-4Rα, human interleukin-4 receptor α subunit; cγ, common receptor γ chain; hcγ, human common receptor γ chain; mcγ, murine common receptor γ chain; hG-CSFR, human granulocyte-colony-stimulating factor receptor; GM-CSF, human granulocyte/macrophage colony-stimulating factor; IL-2, interleukin-2; IL-2Rβγ, interleukin-2 receptor β and γ chain; DMEM, Dulbecco's modified essential medium; FCS, fetal calf serum; G418, Geneticin; MTT, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-tetrazolium; 4G, hIL-4Rα/hG-CSFR hybrid receptor; IgG, immunoglobulin G; PBS, phosphate-buffered saline.

10^6 cells were washed and resuspended in 400 μl of DMEM, 5% FCS containing 50 μg of pKCR derivative and 2 μg of pSV2neo and subsequently subjected to an electric pulse of 250 V at 1500 microfarads. Cells were then kept in DME5M, 8% plus mIL-3 for 48 h before adding G418 to a final concentration of 1 mg/ml. After cultivation in 24-well plates for 2 weeks (medium was renewed twice), G418-resistant cell clones were propagated in 75-ml flasks and further screened for receptor expression by flow cytometry. For cytometric identification of hIL-4Rα expression on transfectants, 10^6 cells were stained with monoclonal antibody X2/45 (final concentration 400 nM) directed against the extracellular domain of hIL-4Rα (20) in 100 μl of PBS for 20 min at 4 °C, incubated with 100 μg/ml fluorescein (dichlorotriazinyl amino fluorescein)-conjugated goat-anti mouse IgG Fcγ fragment-specific (Dianova) and analyzed using a Coulter Epics Elite flow cytometer with a 488-nm laser.

Radioligand Binding Analysis—Iodination of recombinant hIL-4 was done as described (21), specific activity of the radiolabeled cytokine (0, 5 μCi/pmol) was determined by competition binding measurements employing a solid phase binding assay based on the recombinant extracellular domain of hIL-4Rα (6). Saturation binding experiments were performed after a standard protocol (22). Briefly, samples of 2×10^6 cells were incubated with varying concentrations of radioligand in a volume of 200 μl at 4 °C for 2 h. Cells were then separated from unbound radioactivity by centrifugation through a silicon oil layer, and bound label was determined using a γ counter (Beckman). Unspecific binding of radioligand as measured in a parallel experiment, including at least 100-fold excess of unlabeled ligand was subtracted before plotting the data.

Chemical Cross-linking of Radiolabeled hIL-4 to Cells and Immunoprecipitation of Receptor Complexes—Samples of 3×10^7 cells were rinsed twice with RPM1 medium and incubated in 500 μl of RPMI, 2% bovine serum albumin containing 1 nM ^{125}I-labeled hIL-4 (0.5 μCi/pmol) at 4 °C for 1 h. Cells were then washed twice with ice-cold RPMI, treated with 1 mM disuccimidyl suberate (Pierce) in PBS at 4 °C for 30 min, washed with PBS, and subsequently lysed in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 0, 5% Nonidet P-40, 17 mM $Na_2P_2O_7$, 2 mM phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 100 μg/ml leupeptin). After an incubation at 4 °C for 30 min the cell lysates were frozen at −70 °C for 12 h and thawed on ice. Insoluble material was pelleted by centrifugation at 15,000 × *g* for 15 min. Supernatants were gently rotated for 1 h at 4 °C after addition of either 1 ng of anti-hIL-4Rα antibody X14/38,[2] or 0.5 μl of ascites fluid containing anti-mcy antibody TUGm3 (2) (a gift from Dr. K. Sugamura, Tohoku University School of Medicine, Sendai, Japan). Precipitation of immunocomplexes was performed by centrifugation at 2,000 × *g* for 2 min after an additional incubation for 1 h at 4 °C with 12 μl of anti-mouse IgG- (or anti-rat IgG)-agarose slurry (Sigma). The agarose pellets were washed once in lysis buffer, once in 100 mM Tris-HCl, pH 8.0, 0.5 M LiCl, and finally boiled for 5 min in 2 × Laemmli buffer. Pellets were then removed by centrifugation and supernatants subjected to SDS-polyacrylamide gel electrophoresis (6%). After fixing in 12.5% acetic acid, 12.5% 2-propanol for 30 min, gels were dried and subsequently scanned for radioactivity using a PhosphorImager (Molecular Dynamics).

Cytokines, Cell Proliferation Assays, and Data Processing—Wild type hIL-4 and mutants R121D, Y124D, and S125D have been described previously (5, 6). Double and triple mutants R121D/Y124D, R121D/S125D, Y124D/S125D, and R121D/Y124D/S125D, which have been constructed and purified in the same way and are described in detail elsewhere (20). As a source of murine IL-4 the culture supernatant (10%) of an mIL-4 secreting 3T3 transfectant cell line (provided by Dr. W. Müller, Institute of Genetics, University of Cologne) was used. Cytokine-induced proliferation of FDC-4G and TF-1 cells was measured either spectrometrically by means of blue formazan formation from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide-tetrazolium (MTT) or by [^{3}H] thymidine incorporation into *de novo* synthesized DNA as described previously (23). In both cases cells were washed in medium twice to remove mIL-3 or hG-CSF and incubated with 100 or 200 μl of medium containing various concentrations of IL-4 or IL-4 variants for 24 h (FDC-4G cells) or 72 h (TF-1 cells), followed by incubation with MTT or [^{3}H]thymidine for 4 h, respectively.

Statistical evaluations of proliferation and radioligand binding data were done using the computer program GraFit (Erithacus Software).

[2] P. Reusch, unpublished data.

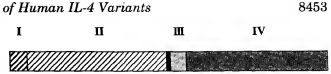


FIG. 1. **Structural elements of the hIL-4Rα/hG-CSFR hybrid receptor.** *Shaded* and *dotted boxes* represent amino acid sequences derived from hIL-4Rα and hG-CSFR, respectively. *Roman numerals* indicate protein domains, the respective numbers of amino acids are given in *arabic numerals*. *I*, signal peptide; *II*, extracellular domain of hIL-4Rα; *III*, transmembrane; *IV*, intracellular domain of G-CSFR. The *black bar* stands for the peptide sequence Leu-Glu equivalent to an *Xho*I restriction site.

RESULTS

Generation of the Murine Myeloid Cell Line FDC-4G Expressing a Chimera of hIL-4Rα and hG-CSFR—A hybrid gene comprising the first 232 codons of the human hIL-4Rα gene (encoding the signal peptide and the extracellular domain) (1) fused to codons 605 through 813 of the hG-CSFR gene (24) (encoding the transmembrane and intracellular domain) was constructed in two consecutive steps. First a *Bam*HI/*Bcl*I fragment was amplified from hIL-4Rα cDNA by polymerase chain reaction with the 5′-primer STA12 (5′-CTAAGGATCCATGGGGTGGCTTTGCTCTG-3′) and the 3′-primer KHF8 (5′-ATGCTGATCAAAGCTTCGATATCTTCTCGAGGTGCTGCTCGAAGGGCTC-3′) and ligated into the unique *Bam*HI site of the expression vector pKCR (14). Primer KHF8 introduced unique restriction sites for both *Xho*I and *Hind*III into the plasmid. They were subsequently used to clone the hG-CSFR gene fragment which was amplified from human placenta total RNA with the primers KHF9 (5′-GAAGATCTCGAGATCATCCTGGGCCTG-3′) and KHF10 (5′-GAAGATCTGATATCTACTCGAGAAGCTTCTAGAAGCTCCCCAG-3′). The resulting construct pKCR-4G encodes a mature hybrid receptor protein of 417 amino acids length, including two codons (Leu-Glu) equivalent to the *Xho*I site at the fusion position (see Fig. 1).

pKCR-4G along with the selection plasmid pSV2neo (15) was introduced into FDC-P1 cells by electroporation. After selection in the presence of G418, a resistant clone was stained with an hIL-4Rα specific monoclonal antibody and was analyzed for surface expression of hIL-4Rα by flow cytometry. The cells from this clone, termed FDC-4G, displayed an approximately 10-fold increase in fluorescence when compared with control cells (Fig. 2), thus clearly indicating a hIL-4Rα-positive phenotype.

FDC-4G Cells Have Acquired Proliferative Responsiveness to Human Interleukin-4—We next examined whether the G418-resistant transfectant clone FDC-4G had become responsive to hIL-4. FDC-P1 cells have been shown to respond to murine IL-4 by transient proliferation (25); therefore we used mIL-4 as a control in the proliferation tests. Fig. 3 shows that although FDC-P1 cells were only responsive to mIL-4, transfectant clone FDC-4G also proliferated in a hIL-4-dependent manner. This was consistent with the cytometry data and confirmed that this cell line functionally expresses the hIL-4α/hG-CSFR hybrid receptor.

Since the G-CSFR can transmit a sustained growth signal into factor-dependent FDC-P1 cells when activated by ligand-induced dimerization (26, 27), we examined if hIL-4 could replace mIL-3 to allow for permanent culture of FDC-4G cells. Cultivation tests over a wide range of hIL-4 concentrations (up to 10 μM) showed that hIL-4, like mIL-4, can prevent cell death for only 72–96 h (data not shown).

Ligand Binding Characteristics of the hIL-4Rα/hG-CSFR Hybrid in the Murine Cellular Background—We next wished to measure the affinity for hIL-4 of the signaling-competent 4G hybrid receptor as well as the number of ligand binding sites

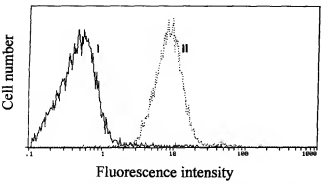


FIG. 2. **Cytometric analysis of G418-resistant transfectant cell clone FDC-4G.** FDC-P1 (*I*) and FDC-4G cells (*II*) were stained with anti-IL-4Rα mAb X2/45 and fluorescein-conjugated anti-mouse IgG and subsequently analyzed by flow cytometry as described under "Material and Methods."

per cell. Therefore the saturation binding experiment shown in Fig. 4 was performed. The K_d was found to be 158 pM, which is in the same range as was determined for human peripheral T cells (5). A saturation of hIL-4 binding to FDC-4G cells was observed at a concentration of 69 pM bound radioligand. This value is equivalent to 3,500 hIL-4 binding sites/cell.

The hIL-4Rα/hG-CSFR Hybrid and Murine cγ Chain Form a hIL-4 Binding Receptor Complex on FDC-4G Cells—Chemical cross-linking of radiolabeled hIL-4 to FDC-4G cells followed by immunoprecipitations using antibodies against IL-4R components was performed in order to define the composition of the operative receptor complex. As shown in Fig. 5, monoclonal antibody X14/38 directed against the extracellular domain of hIL-4Rα predominantly precipitates a radiolabeled complex with a molecular mass of approximately 75 kDa. This band originates from hIL-4 cross-linked to the hIL-4Rα/hG-CSFR hybrid receptor, since it corresponds to the expected size of the glycosylated chimera and does not appear in control precipitations from FDC-P1 cells (data not shown) and TF-1 cells. The latter instead show a dominant band (approximately 155 kDa) which can be attributed to a conjugate of iodinated hIL-4 and hIL-4Rα. Presence of the extracellular domain of hIL-4Rα in both complexes could also be proven by probing Western blots of immunoprecipitations with mAb X14/38 (data not shown).

As a second component, murine common γ chain could be identified in the hIL-4 binding receptor complex on FDC-4G cells. In addition to the hIL-4/4G conjugate, a weaker band running at approximately 80 kDa was precipitated by anti-hIL-4Rα antibody. Due to its proximity to the intense 4G receptor band, it was only resolved on short exposures. This band showed the same migration behavior as the hIL-4/hcγ conjugate from TF-1 cells,[3] and, together with the 4G hybrid derived band, appeared prominently when ^{125}I-hIL-4 cross-linked proteins of FDC-4G cells were immunoprecipitated with mcγ-specific mAb TUGm3. Moreover, both hIL-4Rα- and mcγ-specific antibodies were capable of precipitating a molecular species of approximately 140 kDa in size, which most likely represents the ternary complex hIL-4/4G/mcγ.

Biological Activities of hIL-4 Signaling Mutants on the Murine Cell Line FDC-4G and the Human Cell Line TF-1 Are Strikingly Different—In order to address the activation of the 4G hybrid receptor during ligand binding, we subjected FDC-4G cells to proliferation experiments using a set of hIL-4 mutant proteins. It has been shown previously that hIL-4 variants with amino acid positions Arg^{121}, Tyr^{124}, and Ser^{125}, respectively, exchanged for aspartic acid were affected in their signaling properties on hIL-4-responsive human cells (5, 6, 28). Therefore we employed variants R121D, Y124D, and S125D (6) as well as the double and triple mutants R121D/Y124D, R121D/S125D, Y124D/S125D, and R121D/Y124D/S125D (20) to these tests. The TF-1 cell line expressing the authentic human IL-4R was used as a reference.

The unexpected result was that proliferative activities of individual hIL-4 mutants were drastically different in the two cellular systems. As illustrated in Fig. 6*A*, mutants Y124D and S125D acted like wild type hIL-4 on FDC-4G cells, whereas variant R121D as well as double and triple hIL-4 mutants containing the R for D exchange at position 121 displayed no significant activity. Double mutant Y124D/S125D showed an intermediate, partial agonist phenotype.

It has been demonstrated that hIL-4 Y124D behaves as a high affinity antagonist when assayed in proliferation tests with both human peripheral T cells (5) and TF-1 cells (28). Our results with TF-1 (Fig. 6*B*) were consistent with these findings. In contrast to FDC-4G cells, TF-1 cells respond to Y124D only to a minimal extent (partial agonist activity <5% of wild type). R121D, which was unable to induce FDC-4G proliferation, had some 30% partial agonist activity in the TF-1 system. All double and triple mutants were devoid of any detectable proliferative activity.

An Amino Acid Replacement at Position Arg^{121} Renders hIL-4 Variants Antagonists in FDC-4G Cells—Competition experiments were performed in order to address whether hIL-4 variants with the R121D mutation were inactive on FDC-4G cells because of impaired binding to the heterologous hIL-4R derivative expressed on these cells or due to antagonist properties of the mutant ligands. Fig. 7 shows that R121D as well as the double and triple mutants R121D/Y124D, R121D/S125D, and R121D/Y124D/S125D competitively inhibited hIL-4-induced proliferation of FDC-4G cells. The results obtained with Y124D confirmed that this variant, despite being a potent hIL-4 antagonist for TF-1 cells (28), has agonist activity on FDC-4G cells comparable with that of wild type hIL-4.

DISCUSSION

Human IL-4 receptor α chain has been shown to render murine IL-4-reactive cells responsive to hIL-4 when expressed after gene transfer (8–11). Human and mouse IL-4Rα bind exclusively to IL-4 from the same species *in vitro* (7), hence IL-4Rα obviously mediates species specificity of the activated IL-4 receptor. Functional IL-4R, however, includes at least one additional receptor component that was found to be identical with the interleukin-2 receptor γ chain or cγ (2, 3). hIL-4 responsiveness of murine cells due to expression of hIL-4Rα thus implies that murine cγ can accommodate both mIL-4 and hIL-4.

In order to generate a hIL-4-responsive cell line, we have expressed a chimera of hIL-4Rα and hG-CSGR in FDC-P1 cells. The initial reason for taking this approach was our interest in the functional roles of individual IL-4R subunits. It is only beginning to become understood what particular contributions the cytoplasmic parts of IL-4Rα and cγ make to an IL-4-specific cellular response. Insight into the function of cytoplasmic portions of the IL-4R in the course of signaling is as yet limited. Cytoplasmic sequence elements of hIL-4Rα have been correlated with functional importance in hIL-4 induced proliferation (8, 11, 29). It has been shown that a small sequence motif in the intracellular domain of IL-4Rα is critical for interaction with insulin receptor substrate 1 (IRS-1) protein (29), a molecular contact which probably (among other consequences) leads to an activation of phosphoinositol 3-kinase and apparently links the IL-4R to a signaling pathway with similarities to a set of reactions initiated by the activated insulin receptor. Two phos-

[3] A. Duschl, submitted for publication.

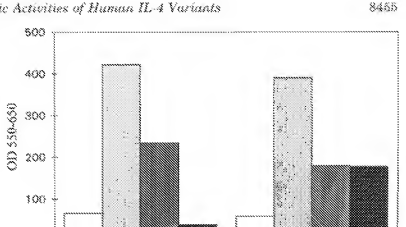


FIG. 3. **Proliferative response of FDC-P1 and FDC-4G cells to mIL-3, mIL-4, and hIL-4.** FDC-P1 cells as well as clone FDC-4G derived from a transfection experiment with the hIL-4Rα/hIL-13Rα hybrid receptor expression construct were tested for cell proliferation by the MTT method as described under "Materials and Methods." Formation of formazan dye was determined spectrometrically after 24-h incubation without cytokine stimulation (*white bars*) or in response to mIL-3 (undiluted culture supernatant of mIL-3 producing X63Ag8 – 6.6.5 cells; *dotted bars*), mIL-4 (undiluted culture supernatant of mIL-4 producing 3T3 cells; *striped bars*), and hIL-4 (2 nM; *black bars*).

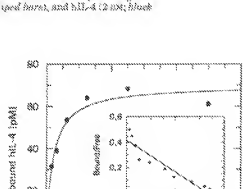


FIG. 4. **Saturation binding of ^{125}I-hIL-4 to FDC-4G cells.** Samples of 3×10^5 FDC-4G cells were incubated with increasing concentrations of radiolabeled hIL-4. Bound label was measured as described under "Materials and Methods." The inset shows a Scatchard plot of the data. Saturation was reached at a bound ligand concentration of 69 pM (43,600 binding sites/cell); K_d was determined to be 166 pM.

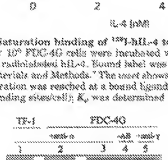
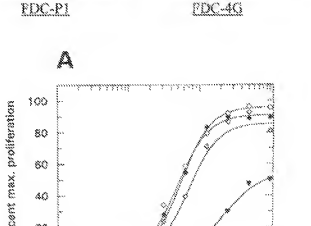


FIG. 5. **Immunoprecipitation of ^{125}I-hIL-4–cross-linked receptor complexes from FDC-4G cells and TF-1 cells.** After chemical cross-linking of radiolabeled hIL-4 to FDC-4G cells or TF-1 cells, immunoprecipitation using the indicated antibodies followed by polyacrylamide gel electrophoresis was performed as described under "Material and Methods." *Lanes 1, 2, and 3*, immunoprecipitations from lysates of TF-1 cells and FDC-4G cells, respectively, with anti-hIL-4Rα antibody X1/28. *Lane 4*, control precipitation from a lysate of FDC-4G cells without receptor antibody added. *Lane 5*, immunoprecipitation from a lysate of FDC-4G cells with anti-mcγ antibody TUGm5. *Lane 2* shows a shorter exposure of an identical sample as in lane 3.



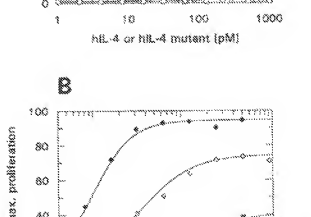


FIG. 6. **Proliferative activity of hIL-4 and hIL-4 mutants on FDC-4G cells and TF-1 cells.** FDC-4G cells (*A*) and TF-1 cells (*B*) were incubated with the concentrations of hIL-4 or hIL-4 mutant indicated. Cell proliferation was determined by [^{3}H]thymidine incorporation after 24 h for FDC-4G or 72 h for TF-1. ♦, hIL-4 wild type; ◇, hIL-4 Y124D; ■, hIL-4 R121D; ○, hIL-4 S125D; ▼, hIL-4 Y124D/S125D; ●, hIL-4 R121D/Y124D; ▽, hIL-4 R121D/S125D; □, hIL-4 R121D/Y124D/S125D.

phosyrosine-containing peptides derived from the intracellular domain of hIL-4Rα were found to directly interact with the IL-4-inducible transcription factor IL-4 Stat (30). In addition,

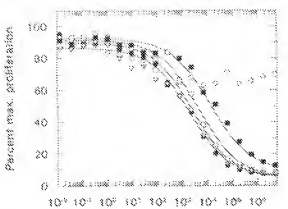


FIG. 7. **Competitive inhibition of hIL-4-induced proliferation of FDC-4G cells by hIL-4 mutants.** Cell proliferation was measured by [^{3}H]thymidine incorporation in the presence of a constant concentration of hIL-4 wild type (2 pM) and increasing concentrations of hIL-4 mutants as indicated. ○, hIL-4 Y124D; ■, hIL-4 R121D; □, hIL-4 R121DY124D; ▼, hIL-4 R121DS182D; ◇, hIL-4 R121DY124DS182D.

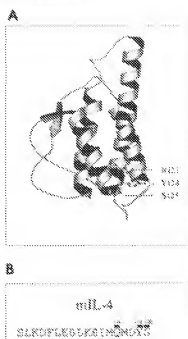


FIG. 8. **Structural situation of hIL-4 amino acids presumably interacting with cγ.** *A*, solution structure of hIL-4 after (35) showing the spatial locations of amino acid side chains involved in signaling. *B*, amino acid alignment of the C termini of mIL-4 (*top*) and hIL-4 (*bottom*). Sequence identities are indicated, and amino acid positions of hIL-4 probably interacting with cγ as well as their murine counterparts are *highlighted*.

assumption of members of the JAK kinase family with the IL-4 receptor complex was demonstrated (31, 32). Recent results obtained on the IL-2R and (in less detail) the IL-4R system indicate an interaction of JAK-3 with cγ and strongly suggest a binding of JAK-1 to IL-4Rα (33–35). Both kinases become phosphorylated as a consequence of ligand-induced receptor activation, whereas only JAK-3 is activated.

The stoichiometry of the IL-4 receptor complex has not yet been definitely settled. By functionally expressing a hIL-4Rα/hG-CSFR chimera we anticipated a system yielding information about oligomerization processes during hIL-4 receptor activation, in particular about a possible involvement of hIL-4Rα homodimerization. G-CSFR has been shown to homodimerize upon ligand binding, and moreover, the intracellular domain of G-CSFR was used experimentally to transmit a homodimerization signal of a fused extracellular human growth hormone receptor domain into FDC-P1 cells, resulting in sustained human growth hormone-dependent cell proliferation (12, 18). The failure of hIL-4 to maintain sustained growth of FDC-4G cells makes a participation of hIL-4Rα homodimerization in formation of the activated hIL-4R appear very unlikely. Together with our chemical cross-linking data it rather confirms the notion that hIL-4 binding leads to an assembly of hIL-4Rα and cγ there, murine cγ into a functional receptor complex, an event which is fundamental for signaling.

FDC-4G cells show a similar growth behavior in response to mIL-4 and hIL-4 regarding both intensity and duration. Since hIL-4 operates by means of a hybrid receptor system taking the intracellular domain of hIL-4Rα, this raises the question what common intracellular mechanisms are involved in both cases. The cytoplasmic portion of G-CSF receptor shares a membrane-proximal box of sequence homology with both IL-4Rα and IL-2 receptor β subunit (36). For IL-2Rβ, a stretch of amino acids comprising this motif was found to be essential for interaction with JAK-1 kinase (32). As for IL-4, IL-2, and several other cytokines, tyrosine phosphorylation of Stat family members and other proteins has also been observed upon G-CSF-induced activation of its cognate receptor, probably due to JAK kinase activity (37). Our recent work indicates that stimulation of FDC-4G cells by hIL-4 results in phosphorylation of the 4G hybrid receptor.[*] We suggest that by interacting with their extracellular domains, hIL-4 brings the intracellular domains of 4G receptor and cγ into close proximity to each other. The serine-rich membrane proximal sequence motif of the hG-CSFR component is associated with a JAK kinase which in turn activates co-associated JAK-3 kinase. As a result of this event a mitogenic signal is generated by the artificial receptor subunit combination which resembles that one produced by the natural murine IL-4R complex.

We finally used the FDC-4G line as a cellular read-out system allowing for the molecular analysis of interactions between hIL-4, hIL-4Rα, and cγ in the course of receptor activation, thereby extending previous mutational analyses of hIL-4 (5, 6). The most surprising results of this study were obtained when the proliferative response of FDC-4G cells and TF-1 cells to signaling-different hIL-4 variants were compared. These data indicate that the participation of human or murine cγ in the formation of a productive hIL-4/hIL-4 receptor complex is not equivalent. Instead, requirements of hcγ and mcγ, respectively, for the integrity of particular amino acid side chains in helix D of hIL-4 differ in a characteristic fashion. Variant hIL-4 R121D displayed no detectable biological activity on murine FDC-4G cells expressing a hIL-4Rα/G-CSFR chimera and behaved as an antagonist in competition experiments with wild type hIL-4. In contrast, when assayed with the human cell line, it showed about 40% wild type activity. For variant hIL-4 Y124D an almost reciprocal activity profile on the two cell types was observed. Although proliferation of FDC-4G cells was induced by Y124D to an extent indistinguishable from that of wild type hIL-4, the variant cytokine had only a minimal residual activity on TF-1.

It is interesting to note that the two most critical amino acids

[*] A. Lischke and R. Friedrich, unpublished results.

of hIL-4 for signaling in FDC-4G and TF-1 cells, respectively (Arg^{121} and Tyr^{124}), are separated by one helical turn of helix D, which results in an aligned spatial location of their side chains (38) (see Fig. 8A). Since sequence comparison reveals a shortened C terminus by four amino acids of mIL-4 compared with hIL-4 (Ref. 7, see also Fig. 8B), it is tempting to speculate that the interaction interface of mIL-4 is shifted by one helical turn equivalent. It has been reported that deletion of the three C-terminal amino acids of mIL-4 result in a variant cytokine with antagonist properties (7). In the light of the findings presented in this study, it would be interesting to analyze mIL-4 Q116D and mIL-4 Y119D, the murine homologs of hIL-4 R121D and hIL-4 Y124D, respectively, with regard to their activity on murine cells. We cannot exclude the possibility that murine and human cγ have different functional characteristics in IL-4 signaling. In the IL-2 receptor system, human IL-2Rβγ heterodimer is capable of binding IL-2, whereas murine βγ is not (39). Biochemical studies on the interaction of IL-4 and cγ are important to address whether such differences also exist regarding the IL-4R.

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REFERENCES

1. Idzerda, R. L., March, C. J., Mosley, B., Lyman, S. D., Vanden Bos, T., Gimpel, S. D., Din, W. S., Grabstein, K. H., Widmer, M. B., Park, L. S., Coeman, D., and Beckman, M. P. (1990) *J. Exp. Med.* **171**, 861–873
2. Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., and Sugamura, K. (1993) *Science* **262**, 1874–1879
3. Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedman, M. C., Miyajima, A., Puri, R. K., Paul, W. E., and Leonard, W. J. (1993) *Science* **262**, 1880–1883
4. Bazan, J. F. (1990) *Immunol. Today* **11**, 350–354
5. Kruse, N., Tony, H.-P., and Sebald, W. (1992) *EMBO J.* **11**, 3237–3244
6. Kruse, N., Shen, B.-J., Arnold, S., Tony, H.-P., Müller, T., and Sebald, W. (1993) *EMBO J.* **12**, 5121–5129
7. Morrison, B. W., and Leder, P. (1992) *J. Biol. Chem.* **267**, 11957–11963
8. Harada, N., Yang, G., Miyajima, A., and Howard, M. (1992) *J. Biol. Chem.* **267**, 22752–22758
9. Skoda, R. C., Seldin, D. C., Chiang, M. K., Peichel, C. L., Vogt, T. F., and Leder, P. (1993) *EMBO J.* **12**, 2645–2653
10. Sakamaki, K., Wang, H.-M., Miyajima, I., Kitamura, T., Todokoro, K., Harada, N., and Miyajima, A. (1993) *J. Biol. Chem.* **268**, 15833–15839
11. Koettnitz, K., and Kalthoff, F. S. (1993) *Eur. J. Immunol.* **23**, 988–991
12. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
14. O'Hare, K., Benoist, C., and Breathnach, R. (1981) *Proc. Natl Acad. Sci. U. S. A.* **78**, 1527–1531
15. Southern, P. J., and Berg, P. (1982) *J. Mol. Appl. Genet.* **1**, 327–341
16. Dexter, T. M., Garland, J., Scott, D., Scolnick, E., and Metcalf, D. (1980) *J. Exp. Med.* **152**, 1036–1047
17. Kitamura, T., Tange, T., Terasawa, T., Chiba, S., Kuwaki, T., Miyagawa, K., Piao, Y.-F., Miyazono, K., Urabe, A., and Takaku, F. (1989) *J. Cell. Physiol.* **140**, 323–334
18. Karasuyama, H., and Melchers, F. (1988) *Eur. J. Immunol.* **18**, 97–104
19. Kruse, N., Lohrabecher, T., and Sebald, W. (1991) *FEBS Lett.* **286**, 58–60
20. Tony, H.-P., Shen, B.-J., Reusch, P., and Sebald, W. (1994) *Eur. J. Biochem.* **225**, 659–665
21. Cabrillat, J., Galizzi, J.-P., Djossou, O., Arai, N., Yokota, T., Arai, K., and Banchereau, J. (1987) *Biochem. Biophys. Res. Commun.* **149**, 995–1001
22. Park, L. S., Friend, D., Grabstein, K., and Urdal, D. L. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1669–1673
23. Duschl, A., Jahn, U., Bertling, C., and Sebald, W. (1992) *Eur. Cytokine Netw.* **3**, 97–102
24. Fukunaga, R., Seto, Y., Mizushima, S., and Nagata, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 8702–8706
25. Carr, C., Aykent, S., Kimack, N. M., and Levine, A. D. (1991) *Biochemistry* **30**, 1515–1523
26. Pub, G., Cunningham, B. C., Fukunaga, R., Nagata, S., Goeddel, D. V., and Wells, J. A. (1992) *Science* **256**, 1677–1680
27. Ishizaka-Ikeda, E., Fukunaga, R., Wood, W. I., Goeddel, D. V., and Nagata, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 123–127
28. Zurawski, S. M., Vega, F., Jr., Huyghe, B., and Zurawski, G. (1993) *EMBO J.* **12**, 2663–2670
29. Keegan, A. D., Nelms, K., White, M., Wang, L.-M., Pierce, J. H., and Paul, W. E. (1994) *Cell* **76**, 811–820
30. Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M., McKnight, S. L. (1994) *Science* **265**, 1701–1706
31. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y.-Q., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W., and O'Shea, J. J. (1994) *Nature* **370**, 151–153
32. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994) *Nature* **370**, 153–157
33. Boussiotis, V. A., Barber, D. L., Nakarai, T., Freeman, G. J., Gribben, J. G., Bernstein, G. M., D'Andrea, A. D., Ritz, J., and Nadler, L. M. (1994) *Science* **266**, 1039–1042
34. Russell, S. M., Johnston, J. A., Noguchi, M., Kawamura, M., Bacon, C. M., Friedman, M., Berg, M, McVicar, D. W., Witthuhn, B. A., Silvennoinen, O., Goldman, A. S., Schmalstieg, F. C., Ihle, J. N., O'Shea, J. J., and Leonard, W. J. (1994) *Science* **265**, 1042–1045
35. Miyazaki, T., Kawahara, A., Fujii, H., Nakagawa, Y., Minami, Y., Liu, Z.-J., Oishi, I., Silvennoinen, O., Witthuhn, B. A., Ihle, J. N., and Taniguchi, T. (1994) *Science* **266**, 1045–1047
36. Murakami, M., Narazaki, M., Hibi, M., Yawata, H., Yasukawa, K., Hamaguchi, M., Taga, T., and Kishimoto, T. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 11349–11353
37. Matsuda, T., and Hirano, T. (1994) *Blood* **83**, 3457–3461
38. Müller, T., Dieckmann, T., Sebald, W., and Oschkinat, H. (1994) *J. Mol. Biol.* **237**, 423–436
39. Kumaki, S., Kondo, M., Takeshita, T., Asao, H., Nakamura, M., and Sugamura, K. (1993) *Biochem. Biophys. Res. Commun.* **193**, 356–363